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An intrinsic vasopressin system in the olfactory bulb is involved in social recognition

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Abstract

Many peptides, when released as chemical messengers within the brain, have powerful influences on complex behaviours. Most strikingly, vasopressin and oxytocin, once thought of as circulating hormones whose actions were confined to peripheral organs, are now known to be released in the brain where they play fundamentally important roles in social behaviours¹. In humans, disruptions of these peptide systems have been linked to several neurobehavioural disorders, including Prader-Willi syndrome, affective disorders, and obsessive-compulsive disorder, and polymorphisms of the vasopressin V1a receptor have been linked to autism^{2,3}. Here we report that the rat olfactory bulb contains a large population of interneurons which express vasopressin, that blocking the actions of vasopressin in the olfactory bulb impairs the social recognition abilities of rats, and that vasopressin agonists and antagonists can modulate the processing of information by olfactory bulb neurones. The findings indicate that social information is processed in part by a vasopressin system intrinsic to the olfactory system.

Complex social behaviour often depends on individual recognition, and most mammals distinguish individuals by their olfactory signatures. Some individuals are accorded a particular status, such as when a bond is formed between a mother and offspring, or between sexual partners in monogamous species. In these cases, an olfactory memory is forged in the olfactory bulb, partly as a result of the actions of peptides⁴. For example, oxytocin released in the mother's brain during parturition helps to establish the olfactory signatures of the offspring as memorable⁵.

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Author Contributions: VAT and HH contributed equally. ML, GL, and ME designed the experiments. VAT performed the immunohistochemistry and *in vitro* electrophysiology as shown in Fig. 1. DWW, SLM and CC performed tracer injections (Fig. 1m-o). ME and JN performed receptor antagonist and siRNA study with behavioural analysis as shown in Figs 2b,c and 3 as well as suppl. Figs 1, 2a,b. YT and TO produced TG diphtheria toxin rats and performed behaviour experiments shown in Figs 2d,3c and suppl. Fig 2d-i. KL performed *in situ* hybridisation and RL the vasopressin radioimmunoassay. HH performed *in vivo* electrophysiology shown in Fig.4 and suppl. Fig 3. ML, ME, TO, GL and HH performed general data processing and statistical analyses. ML and GL wrote the paper.

Supplementary Information accompanies the paper on www.nature.com/nature

The converse of social attachment is rejection of, or aggression towards, individuals who are recognised as intruders or competitors⁶. For this, vasopressin, a peptide closely related to oxytocin, is important via its actions at V1 receptors, and mice without a functional accessory olfactory system display many of the same behavioural deficits as mice that lack V1 receptors. This suggests that vasopressin is involved in the processing and/or integration of olfactory stimuli, and that it couples socially relevant olfactory cues with an appropriate behavioural response⁷.

Here, we have identified a hitherto unreported population of vasopressin neurones in the olfactory bulb (Fig.1). We first saw these cells in a transgenic rat line in which enhanced green fluorescent protein (eGFP) was targeted to the vasopressin secretory pathway, resulting in its co-packaging with vasopressin in secretory vesicles⁸. The main olfactory bulb contains similar numbers of eGFP-expressing cells in males and females (99 ± 14 and 103 ± 10 cells/section; $n=16,16$), giving an estimated 5,000-7,000 neurones per bulb; the accessory bulbs contained $\sim 1,000$ neurones. These large ovoid neurones ($\sim 15 \mu\text{m}$ diameter) are mostly located in the external plexiform layer close to the glomeruli (the structures in the bulb that directly receive inputs from olfactory receptor cells). Each has several large dendrites, one of which penetrates a single glomerulus where it gives rise to many small branches, suggesting that they receive direct inputs from olfactory nerve afferents. Other dendrites travel laterally to the external zones around neighbouring glomeruli (Fig.1b). By immunocytochemistry, we showed that these cells indeed synthesise vasopressin (Fig.1d), and we confirmed their presence in wild-type rats (Fig.1e). We also confirmed that they express vasopressin mRNA by *in situ* hybridisation (Fig.1f), and that vasopressin is released from olfactory bulb explants *in vitro* in response to depolarisation with high K^+ (release increased from 0.65 ± 0.19 to $4.88 \pm 1.88 \text{ pg/sample}$, $P < 0.01$, $n=9$). The total bulb vasopressin content was $42.9 \pm 2.6 \text{ pg/mg}$ wet weight ($n=12$, mixed sex).

Unlike periglomerular cells and short axon cells (two other cell populations in the same region) the vasopressin cells are immuno-negative for GABA, calretinin and calbindin-D-28K9 (Fig.1g-i), but like external tufted (ET) cells^{9,10} they are immunoreactive for glutamate (Fig.1j-l). No cells were immunoreactive for oxytocin. Whole-cell patch-clamp recordings from olfactory bulb slices showed that the vasopressin cells have electrophysiological characteristics like those of ET cells¹⁰. They show spontaneous bursts of action potentials (1.5 bursts/s) arising at the start of a slow depolarising potential envelope ($6.5 \pm 0.5 \text{ mV}$; $n=5$) from a resting membrane potential of $-55 \pm 2 \text{ mV}$, and have an input resistance of $189 \pm 38 \text{ M}\Omega$. This bursting is voltage-dependent (Fig.1s), and injection of depolarising current converts bursts of action potentials to an irregular firing pattern.

Unlike most ET cells, most vasopressin cells do not project outside the olfactory bulb. Microinjections of the retrograde tracer fluorogold into the cortical amygdala, the piriform cortex or olfactory tubercle (major projection sites of olfactory bulb efferents¹¹) resulted in labelling of most mitral cells (the main output neurones), but no vasopressin cells (Fig.1m-o), injections into the anterior olfactory nucleus produced a very small number of labelled cells (not shown). If the vasopressin cells do not project outside the olfactory bulb, any effects of the vasopressin that they release on olfactory information flow must be reflected by changes in the activity of other output cells. Vasopressin may be released from their axon terminals, but the dendrites may be a more important source as these are densely filled with vasopressin, and in the hypothalamus, vasopressin is released from dendrites in both an activity-dependent manner and in an activity-independent way by agents that mobilise intracellular calcium¹².

Vasopressin receptors are widespread in the main and accessory olfactory bulbs¹³. We found no immunoreactivity for V1a receptors on vasopressin cells, but some for V1b

receptors (Fig.1p-r), so vasopressin may act as an autocrine regulator through this receptor subtype¹⁴. Many other cells in the periglomerular region were immunoreactive for both subtypes, as were mitral cells in the main and accessory bulb.

We tested the hypothesis that olfactory bulb vasopressin is involved in social recognition. It has already been shown that infusion of vasopressin into the bulb can enhance social recognition in rats. In those experiments, a peptide V1 antagonist had no significant effect.¹⁵ Here, we used a non-peptide V1 antagonist (OPC 21268) that diffuses more readily, and which is effective in antagonising the actions of dendritically released vasopressin in the hypothalamus¹⁶. We injected the antagonist bilaterally into the olfactory bulb and tested social discrimination¹⁷. In this test, a juvenile rat is placed in the home cage of an adult male, and the time that the adult spends investigating it is measured. Later, the same juvenile and an unfamiliar juvenile are introduced into the cage. Normally, the adult investigates the familiar juvenile only briefly, and pays most attention to the unfamiliar juvenile; this memory is short lasting (<40min) and is based on olfactory characteristics. In these experiments, the adults were given a microinjection of the antagonist just before the juvenile was first presented. When retested, the adults did not discriminate between the familiar and unfamiliar juveniles, indicating no memory of the juvenile ($F(3,39)=3.34$, $P=0.026$; Fig. 2a,b).

To specifically test involvement of the V1a receptor subtype, we used infusions of a small interference RNA (siRNA) targeted against V1a receptor mRNA (siRNA has been previously used to successfully silence gene expression, including silencing the V2 receptor in mouse kidney¹⁸); these infusions produced transfection in the olfactory bulb but not in the septum (supplementary Fig.1). The effects of siRNA treatment were similar to those obtained with antagonist (treatment: $F(1,16)=17.86$, $P<0.01$; factor interaction: $F(3,48)=4.37$, $P<0.01$, Fig.2c). Control rats (but not siRNA-treated rats) could recognize juveniles by their complex individual olfactory fingerprint even in the presence of distracting mono-molecular odours (supplementary Fig.2a). Treatment with siRNA impaired habituation/dishabituation to juvenile cues (control: $F(4,32)=3.42$, $P<0.02$; siRNA: $F(4,32)=0.47$, $P=0.76$), but not to volatile odours (control: $F(4,32)=5.672$, $P<0.01$; siRNA: $F(4,32)=4.09$, $P<0.01$) or object recognition (Fig.3a-c), and did not affect open field behaviour (supplementary Fig.2b,c).

We also used a transgenic rat line in which the human diphtheria receptor is inserted into the vasopressin promoter region. In these rats, infusion of diphtheria toxin^{19,20} results in a local, selective destruction of vasopressin cells. Transgenic rats pretreated with toxin infusions into the olfactory bulb showed a similar impairment of juvenile recognition (treatment: $F(1,29)=14.95$, $P<0.01$; factor interaction: $F(1,29)=4.99$, $P=0.033$, Fig.2d), again with no impairment of object recognition (Fig.3c), locomotor activity or anxiety-related behaviours (supplementary Fig.2d-i).

Finally, we investigated vasopressin-dependent changes in olfactory bulb output. Current theories of glomerular function propose that olfactory nerve afferents activate ET cells, which activate short axon cells and periglomerular interneurons of the same glomerulus. This amplifies the olfactory nerve input and imposes on it a bursting pattern¹⁰, and this signal is transmitted to mitral cells. We recorded, in freely-breathing anaesthetised rats, from mitral cells antidromically identified by electrical stimulation of the lateral olfactory tract (Fig.4a,b and supplementary Fig.3). Under urethane anaesthesia, most mitral cells display patterned discharge comprising prolonged intermittent bursts of action potentials²¹ (burst duration 159 ± 10 s; interburst time 97 ± 13 s, intraburst firing rate 6.4 ± 0.5 Hz; $n=94$; supplementary Fig. 3). Within these bursts, the firing activity is modulated by the respiratory rhythm. In addition, many mitral cells (57/94) display a bimodal interspike interval

distribution, reflecting the frequent occurrence of spike doublets within bursts. Thus, within bursts, these cells fire at two distinct instantaneous frequencies; at 100-250 Hz (doublets; mean modal interspike interval 3.2 ± 0.4 ms), and at ~50 Hz (mode 18 ± 1 ms). The doublets are noteworthy as it is believed that only high frequency firing episodes are back-propagated into the distal dendrites²². Topical administration of vasopressin or the V1 antagonist onto the exposed bulb dorsal to the recording site modified the electrical activity of mitral cells. Vasopressin reduced the proportion of time they were active, and particularly reduced doublet firing, whereas the antagonist had the opposite effect ($P < 0.05$, paired *t*-tests; Fig.4c-e). In sixteen experiments, each involving a long recording from an identified mitral cell, we identified an odour to which that cell was particularly responsive, established the repeatability of that response in basal conditions, and then retested the response to stimulation after topical application of vasopressin. In every case the response to the odour was suppressed after vasopressin, whereas topical application of aCSF had no effect on the responses of seven cells tested (Fig.4f-j).

These findings suggest that vasopressin is a retrograde signal that filters activation of the mitral cells. Its effects may involve presynaptic modulation of noradrenaline or acetylcholine release, both of which are increased by retrodialysis of vasopressin in the olfactory bulb of ewes²³. Because this filtering is important for social recognition, it seems that the vasopressin release must depend on previous olfactory experience. In the hypothalamus, activity-dependent dendritic vasopressin release can be conditionally regulated ('primed') by recent experience^{12,24}; such a mechanism in the olfactory bulb may thus mediate conditional changes in olfactory recognition.

Thus the olfactory bulb contains vasopressin cells that process olfactory signals relevant to social discrimination, and dendritic vasopressin release may be involved in filtering out familiar signals. Genetic variations in brain vasopressin signalling are associated with differences in social behaviours in humans^{25,26} as well as in animal models. We are not suggesting that social recognition in humans depends on olfactory signals; vasopressin affects social behaviour at many other sites as well as the olfactory bulb^{27,28}, and in humans olfactory recognition probably has only a small role. However, these studies suggest a mechanism by which experience-dependent vasopressin release can facilitate social recognition, and this mechanism may be common to several sites of action.

Methods summary

Animal experimental procedures were conducted with regulatory approval and ethics committee approvals in the UK, Germany and Japan.

Brains were processed immunocytochemically to detect cells expressing vasopressin GFP; calbindin D-28K calretinin; GABA; glutamate; and V1a receptors, and by *in situ* hybridization to detect vasopressin mRNA and eGFP mRNA. Eleven eGFP rats were stereotactically microinjected with the retrograde tracer Fluorogold at various sites to detect cells projecting from the olfactory bulb. Vasopressin content and potassium stimulated release from olfactory bulb explants was measured by RIA.

To test effects on social discrimination, the V1 antagonist or vehicle was infused bilaterally into the olfactory bulbs of adult rats¹⁵. A juvenile was introduced into the adult's cage for 4min, and the duration of investigation by the adult recorded; 30min (or 180min) later, the juvenile was re-introduced with another unfamiliar juvenile, and the preference index was measured¹⁷ (time investigating unfamiliar/(time investigating familiar + time investigating unfamiliar juvenile)*100). Olfactory habituation and dishabituation²⁹ was tested by exposing rats to four 1-min trials separated by 10-min. During a fifth dishabituation trial the

rats were exposed to a novel stimulus. In rats injected with siRNA directed against V1a receptors (or control vectors), behaviours were tested 4, 8 and 16 days after injection.

For conditional ablation of vasopressin neurones, we used transgenic rats with a mutated human heparin-binding epidermal growth factor-like growth factor (hHB-EGF)30 gene under the control of vasopressin promoter. Diphtheria toxin was microinjected into the olfactory bulb in rats anesthetized with tribromoethanol.

In urethane-anaesthetised rats, electrical activity of mitral cells was recorded before and after administration of vasopressin or V1 antagonist onto a small exposure of the bulb. Cells were tested with odours applied for 2s in an airstream directed at the nose. For *in vitro* electrophysiology, whole-cell current-clamp recordings were made from GFP-expressing cells in 300µm horizontal olfactory bulb slices.

Methods

Immunohistochemistry

Brains of eGFP and wild-type Sprague-Dawley rats were perfusion fixed with 4% paraformaldehyde, post-fixed and cryoprotected³¹. Free-floating frozen bulb sections (52µm) were immunostained with primary antibodies (ABs) (see supplementary table) for 24-48h at 4°C. Immunoreactivity was visualised with either biotinylated secondary ABs and Alexa fluorophore conjugated streptavidin or Alexa fluorophore conjugated secondary ABs, at 1:500 for 2h at room temperature. Sections were imaged as previously³¹.

In situ hybridisation

Six adult Wistar rats were decapitated under deep isoflurane anaesthesia. Brains were frozen at -40°C in methylbutane and stored at -80°C. Cryosections (16µm) were thaw-mounted on RNA-free slides and stored at -80°C. *In situ* hybridization was performed on sagittal sections using [³⁵S]-UTP-labelled ribonucleotide probes³². The pcDNA3 plasmid, containing vasopressin precursor cDNA (595bp insert), was linearized with XbaI; the pBluescript KS plasmid, containing the vasopressin specific 3' end (230bp insert) was linearized with HindIII. Both were *in vitro* transcribed with T7 RNA Polymerase to yield an antisense cRNA probe. To generate an eGFP specific riboprobe, the cDNA encoding GFP was subcloned into pBluescript SK. The eGFP fragment of pEGFP-N3 (Clontech, Palo Alto, CA) was isolated using the BamHI and NotI sites and subcloned via the same sites into pBluescript SK. The construct was verified by sequencing. For the eGFP riboprobe, this plasmid was linearized with BamHI and *in vitro* transcribed with T3 polymerase. Specific activity of probes was ~10⁸ cpm/µg. Radioactivity was adjusted to 3,3×10⁶ cpm/100µl hybridization buffer. Slides were dipped in Kodak NTB emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with 0.5% glycerol and exposed for 6 days at 4°C, developed and counterstained³².

Vasopressin release

Sprague-Dawley rats (100-200g) were decapitated and one half of an olfactory bulb was incubated in oxygenated aCSF. 50mM K⁺ was applied for 5min and vasopressin measured in 5-min samples. The other half was sonicated (5×4s), centrifuged (10,000g, 5min) and vasopressin content determined in the supernatant by radioimmunoassay (sensitivity 0.1pg, cross-reactivity to related peptides including oxytocin <0.7%, intra- and inter-assay variability 6-9%).

Antagonist, siRNA and diphtheria toxin injection and behaviour studies

The V1 antagonist³³ (60ng in 3µl aCSF, OPC 21268, Otsuka America Pharmaceutical Inc., Rockville, MD) or aCSF was infused under brief isoflurane anaesthesia over 2min through bilateral guide cannulae (7mm anterior to bregma, 1.3mm lateral, 3mm deep, implanted 5 days before) as previously¹⁵. Rats received both vehicle and antagonist in a balanced crossover design. For object recognition tests³⁴ rats were exposed to a 500ml lab glass bottle (Schott, Mainz, Germany) or a tube rack (W. Krannich GmbH, Göttingen, Germany) for 4min. This was removed and 30min later was re-introduced for 4min with the other object (the preference index was calculated as above). For volatile scent habituation, a stainless steel tea egg (50 × 35mm) hanging from the top of the cage and containing filter paper soaked with 5µl carvone ((R)-(-)-carvone diluted 1:100 in diethylphtalat; Merck, Ismaning, Germany) was presented in trials 1-4. For dishabituation, the filter paper was soaked with 5µl carvone mixed with isoamylacetate. For juvenile habituation, the same juvenile was presented during trials 1-4 and a novel juvenile was used during the dishabituation test. All behaviours were measured by experimenters blind to identity of treatment.

Social discrimination with an additional scent was tested by scenting the juvenile with carvone (5µl; (R)-(-)-carvone diluted 1:100 in diethylphtalat; Merck, Ismaning, Germany) during the first session, and with isoamylacetate (5µl, Sigma-Aldrich, Taufkirchen, Germany; diluted 1:100 in diethylphtalat) in the second session; the novel juvenile was scented with carvone. Open field behaviour was tested in two different laboratories using different equipment. In both cases the rats were naïve to the apparatus and the exposure duration was 10min; exposure started by placing the rat in one corner of the apparatus. Behaviour was recorded with a video camera mounted over the open field and behaviour was scored. Rats (Sprague Dawley) that received the siRNA were placed in an open field apparatus (100×100×50cm) 19 days after treatment. Locomotor activity was scored by counting the number of crossed lines on a grid (10×10cm) on the floor of the open field. Anxiety-related behaviour was monitored by measuring the duration the rat spent within the centre area (80×80cm), expressed as % of the total duration of the open field testing. Transgenic rats (Lewis rats with a mutated human heparin-binding epidermal growth factor-like growth factor (hHB-EGF) gene under the control of vasopressin promoter, see below) were tested 15-18 days after treatment with diphtheria toxin in an open-field apparatus measuring 60×60×40cm. The total distance travelled and the time spent in the centre area (40×40cm) were recorded. The time spent in the centre area was expressed as % of the total duration of testing. Transgenic rats were tested 16-19 days after treatment with diphtheria toxin in the elevated plus maze. The apparatus had two open (50×10cm) and two enclosed arms of the same size with 40cm-high transparent walls (O'Hara & Co, Tokyo, Japan). The arms and central square were constructed of black plastic and elevated to 65cm. The rat was placed on the central platform with its head facing a closed arm. Entries into open arms, time spent in open arms and locomotor activity were measured the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image/>) and Image J program (<http://rsb.info.nih.gov/ij/>). (O'Hara & Co.)³⁵.

siRNA treatment

The purified SMARTvector™ siRNA lentiviral particles targeting the rat vasopressin V1a receptor (No. NM053019; No. SH-092092-02-10) and SMARTvector™ empty vector control particles (No. S-004000-01) were from Thermo Fisher Scientific (Lafayette, CO, USA). The titer of all solutions was >10⁸ transforming Units/ml. Lentiviral solution (2µl) was infused via a fused silica capillary into each olfactory bulb. Behaviour was tested 4, 8 and 16 days later.

To confirm transfection by the SMARTvector™ lentiviral particles, the TurboGFP reporter protein was visualised immunohistochemically at 20 days after injection. Rats were anaesthetised using a mixture of 0.1ml/ 0.06ml Ketavet and Dormitor (Pharmacia GmbH, Erlangen; Bayer Vital GmbH, Leverkusen, respectively, Germany) and transcardially perfused with physiological saline for 2min followed by 4% formaldehyde diluted in 0.1M phosphate buffer pH7.4 (PB). Brains were postfixed for 20h in 4% formaldehyde diluted in PB, transferred to 0.5M sucrose in PB for 6h and to 1M sucrose in PB for ~ 40h. Brains were shock-frozen in isopentanol at -50 °C and stored at -80 °C until cryo-sectioning. Floating frontal sections (25µm) were incubated with anti-TurboGFP antibody (evrogen, Moscow, Russia, Cat # AB511, dilution 1:10000). Immunoreactive cells were visualised using the ABC-method using Axiovision software (Zeiss, Jena, Germany) and counterstained with Nissl staining. Immunostaining was observed in cells that surrounded the track of the injection cannula (not shown) and in cells and fibers in the glomerular, epiform plexus and mitral cell layers of the bulb.

Conditional ablation of vasopressin neurones

We used transgenic Lewis rats with a mutated human hHB-EGF gene under the control of vasopressin promoter³⁶. The hHB-EGF mutant has a high affinity to diphtheria toxin but no EGF-like activity³⁰. Diphtheria toxin binds to the extracellular domain of HB-EGF, is incorporated into cells by receptor-mediated endocytosis, inactivates eukaryotic elongation factor 2 through ADP-ribosylation, and thereby inhibits protein synthesis leading to cell death¹⁹. Diphtheria toxin (D0564, Sigma, 300pg/3µl) was microinjected bilaterally into the bulb of transgenic or wild-type rats anaesthetized with tribromoethanol (200mg/kg i.p.). Transgenic rats injected i.c.v. with the toxin showed polyuria consistent with diabetes insipidus, but no polyuria was seen in rats after intrabulbar injections, indicating no significant loss of hypothalamic vasopressin cells (data not shown).

Retrograde tracing

eGFP rats were stereotactically microinjected under Hypnorm-Hypnovel anaesthesia (Pharmaceuticals, Oxford, UK) with Fluorogold (1µl, 1%, Lumafluor Inc, New City, NY, USA) into the cortical amygdala (-2.1mm posterior to bregma; 4mm lateral; 9.2mm deep; n=3)³⁷, anterior olfactory nucleus (+3.2; +1.5; -8; n=2), piriform cortex (+0.7; +4; -8; n=4) or olfactory tubercle (+2.7; +2; -8; n=2) over 20min. One week later, rats were perfused transcardially with 4% paraformaldehyde and brains processed for immunohistochemistry.

Electrophysiology

For *in vitro* electrophysiology, glass microelectrodes (8–12MΩ) were filled with (mM): K-gluconate 134, MgCl₂ 4, HEPES 10, Mg₂ATP 3 Tris-GTP 0.3 NaCl 4, and EGTA 0.2 (pH7.3 adjusted with KOH, 270mOsm). In urethane-anaesthetised Sprague-Dawley rats (250–300g), extracellular recordings were made using glass microelectrodes filled with 0.9%NaCl (tip diameter ~1µm). Recordings were made from single mitral cells in the main olfactory bulb (~7mm anterior to bregma, ~1.3mm lateral) identified antidromically (collision test) by electrical stimulation of the ipsilateral lateral olfactory tract²¹ (1.4mm posterior to bregma, 3.2mm lateral, 9.5mm deep, latencies in range 1.0–5.8ms)³⁷. Activity quotients (proportion of time active relative to total time) were calculated, and the hazard function (5-ms bins; hazard in bin $[t, t+5]$) = (number of intervals in bin $[t, t+5]$) / number of intervals of length $> t$ plotted³⁸. Odours were applied via a polythene cannula (0.1mm diameter) placed 3mm in front of the nose. Cells were tested with a range of odours including heptanal, hexanal, lemon and peppermint, using a 1-ml syringe filled with odour-saturated air; 0.1ml air was ejected over 2s for each test; clean air had no effect.

Statistics

Data were analysed by ANOVA followed by Fisher's least significant difference test for planned comparisons, Mann-Whitney *U*-test or unpaired Student's *t*-tests. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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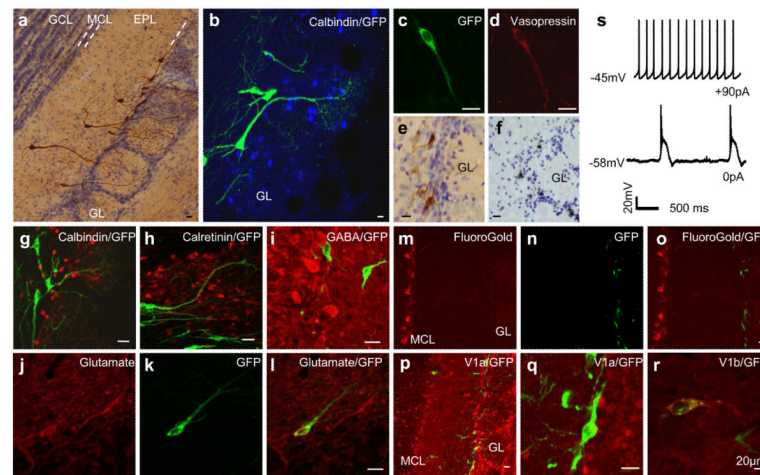


Figure 1. Vasopressin neurons in the olfactory bulb

a, Most vasopressin cells in eGFP transgenic rats (DAB staining) are in the periglomerular region throughout the main olfactory bulb. **b**, An apical dendrite ramifies into a glomerulus (blue staining, periglomerular cell marker calbindin 28K). Confirmation using antibodies against **(c)** GFP and **(d)** vasopressin in transgenic rats and **(e)** vasopressin in wild-type rats. **f**, *in situ* hybridisation for vasopressin mRNA. **g-i**, Vasopressin cells do not co-express calbindin, calretinin or GABA (red), but **(j-l)** contain glutamate. **m-o**, FluoroGold labelling after injection into the anterior olfactory nucleus in mitral and periglomerular cells, but not vasopressin cells. V1a receptors are expressed on **(p)** mitral cells and many periglomerular neurones, but not on **(q)** vasopressin cells, whereas some vasopressin cells express V1b receptors **(r)**. **s**, Patch-clamp recording indicate firing patterns (spontaneous and depolarized) like those of ET cells¹⁰. GCL granule cell layer, MCL mitral cell layer, EPL epiform plexus layer, GL glomerular layer. Scale bars 20µm.

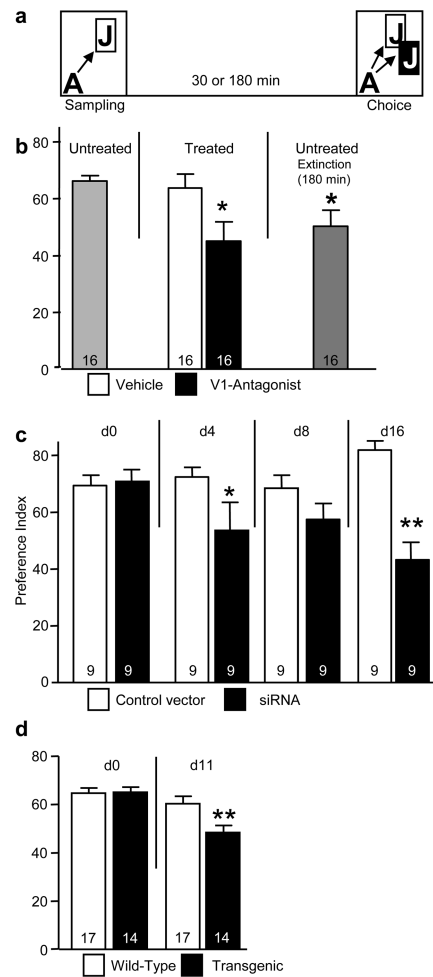


Figure 2. Effects of V1a receptor blockade and vasopressin cell destruction on social recognition
a, A juvenile (J) is presented to an adult male (A) for 4min. This juvenile is removed and after 30 or 180min is re-presented together with a non-familiar juvenile, and the preference index calculated. **b**, administration of V1 antagonist results in performance similar to that after extinction of short-term discrimination (after 180min). **c**, V1a receptor siRNA similarly impairs discrimination after 4, 8 and 16 days of treatment. **d**, Selective destruction of vasopressin cells via diphtheria toxin injection results in a similar impairment of discrimination in transgenic rats, but not in wild-type rats. Means±S.E.M., * $P<0.05$ and ** $P<0.001$ vs control; numbers in columns are n/group.

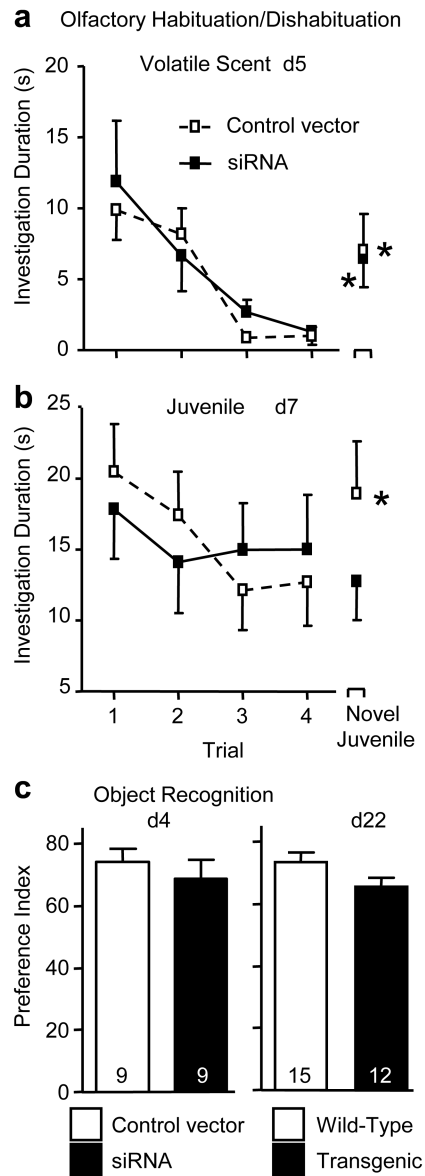


Figure 3. Specificity of effects on social recognition

a, Control rats and siRNA-treated rats showed similar habituation and dishabituation to volatile scents. **b**, Control rats also showed habituation and dishabituation to juveniles, siRNA-treated rats showed neither. **c**, Neither siRNA nor diphtheria toxin injection affected object recognition (**c**). Means+S.E.M., * $P < 0.05$ vs trial 4 and same treatment; numbers in columns are n/group.

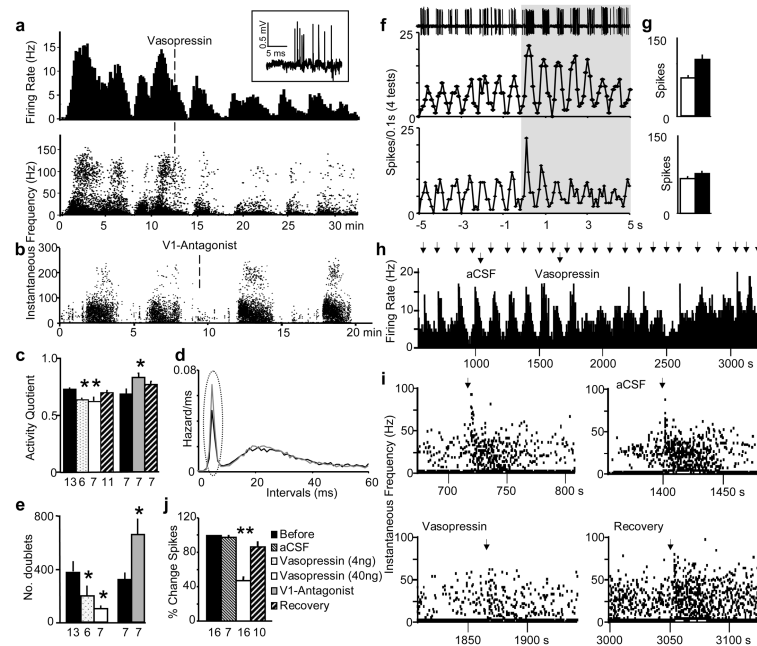


Figure 4. Vasopressin effects on mitral cells

Effects of (a) vasopressin and (b) V1 antagonist on firing rate (a) and instantaneous frequency in a single, representative mitral cell (a,b). The inset in a shows raw waveform traces of spike activity. c, Vasopressin reduced the activity quotient in 6 cells treated with 4ng and 7 treated with 40ng vasopressin, and increased it in 7 cells treated with antagonist (d) the hazard function overlay shows the reduction in doublet firing in a typical cell (light line before, heavy line after vasopressin) (e) change in number of doublets (intervals<10ms); quantified for all doublet cells f, top; spike activity in a mitral cell, showing activity modulated by respiratory rhythm 5s before and after odour; below, cumulative spikes from four tests during bursts before (above) and after (below) vasopressin. g, number of spikes 5s before and 5s after odour for the tests in (f). h, responses of another cell to odour (arrows) before, during and after aCSF and vasopressin. i, expanded instantaneous frequency records of the cell shown in (h). j, Mean odour response in 16 cells tested with vasopressin, 7 of which were also tested with aCSF, and in 10 of which recordings were maintained long enough to observe recovery. Means+S.E.M. * $P<0.05$.